APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2004, p. 4004–4011 Vol. 70, No. 7
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.7.4004–4011.2004

Effect of Pyocyanin on a Crude-Oil-Degrading Microbial Community

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Received 21 October 2003/Accepted 2 April 2004

Pseudomonas aeruginosa **is an *n*-alkane degrader that is frequently isolated from petroleum-contaminated sites and produces factors that enhance its competitiveness and survival in many environments. In this study, one such factor, pyocyanin, has been detected in an oil-degrading culture containing *P. aeruginosa* and is a redox-active compound capable of inhibiting microbial growth. To examine the effects of pyocyanin further, an oil-degrading culture was grown with and without 9.5 μM pyocyanin and microbial community structure and oil degradation were monitored for 50 days. Denaturing gradient gel electrophoresis (DGGE) analysis of cultures revealed a decrease in the microbial community diversity in the pyocyanin-amended cultures compared to that of the unamended cultures. Two members of the microbial community in pure culture exhibited intermediate and high sensitivities to pyocyanin corresponding to intermediate and low levels of activity for the antioxidant enzymes catalase and superoxide dismutase, respectively. Another member of the community that remained constant in the DGGE gels over the 50-day culture incubation period exhibited no sensitivity to pyocyanin, corresponding to a high level of catalase and superoxide dismutase when examined in pure culture. Pyocyanin also affected the overall degradation of the crude oil. At 50 days, the culture without pyocyanin had decreased polycyclic aromatic hydrocarbons compared to the pyocyanin-amended culture, with a specific reduction in the degradation of dibenzothiophenes, naphthalenes, and C_{29} and C_{30} hopanes. This study demonstrated that pyocyanin influenced the diversity of the microbial community and suggests the importance of understanding how interspecies interactions influence the degradation capability of a microbial community.**

Degradation of crude oil in the environment by autochthonous microbial communities has been well documented, and individual microorganisms capable of metabolizing components of crude oil have been isolated from a variety of ecosystems (2, 28, 32). Individual microorganisms are capable of degrading only a limited number of crude oil components; thus, more extensive degradation of oil depends on the presence of metabolically diverse microbial communities (2, 8, 18, 21, 29, 39). However, microbial communities are not static, and factors affecting the composition of the community will alter their degradation potential. Alterations in community degradation potential due to physicochemical changes (i.e., temperature, nutrient availability) have been characterized (4, 6, 14, 37). However, little is known about the connection between crude oil and microbial interspecies interactions and how this influences the overall degradation capacity of the community.

Microbial community profiles shift as a result of environmental exposure to crude oil because of the growth and loss of microorganisms capable and incapable of metabolizing crude oil components (19, 41). However, within populations of crude oil-degrading microorganisms, interactions resulting from each microorganism's metabolic capability can be synergistic or detrimental to further oil degradation (3, 13). For example, the production of rhamnolipid biosurfactants by *Pseudomonas aeruginosa* has been shown to enhance the rates of octadecane (46) and hexadecane (1) uptake and degradation. However, it has also been demonstrated that the presence of *P. aeruginosa* isolates in crude oil-degrading enrichment cultures decreases microbial diversity and aromatic degradation (21). In the later study, two oil-degrading microbial communities were initiated with soil from a polycyclic aromatic hydrocarbon (PAH)-contaminated site (20, 21). One community was enriched in the laboratory on oil containing *n*-alkanes, while the second was enriched on oil lacking *n*-alkanes. The culture developed in the presence of *n*-alkanes was characterized by reduced microbial diversity, the presence of *P. aeruginosa*, and substantially reduced PAH degradation, while the culture developed in the absence of *n*-alkanes contained a more diverse community, lacked the presence of *P. aeruginosa*, and was capable of aromatic degradation. Thus, the presence of *P. aeruginosa* in crude oil-degrading cultures may influence microbial community diversity, hence influencing the efficiency of crude oil degradation.

P. aeruginosa is frequently isolated from petroleum-contaminated sites and is capable of producing metabolites (i.e., alginate, rhamnolipid, pyocyanin) that enhance its competitiveness and survival (24, 46). For example, the water-soluble secondary metabolite pyocyanin (1-hydroxy-5-methylphenazine) has demonstrated antimicrobial activity against a variety of microorganisms (5, 24, 33). While the direct mechanism of pyocyanin toxicity remains unclear, the wide range of biological activity is thought to be due to its ability to catalyze the formation of toxic radicals such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (24). It has been proposed that microorganisms expressing high levels of the antioxidant enzymes catalase and superoxide dismutase (SOD) could tolerate pyocyanin more effectively than microorganisms not expressing or expressing low levels of the enzymes (5, 24, 25). Further, it is thought that pyocyanin production may be coupled to intracellular ATP

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levels, resulting in increased pyocyanin production under carbon- or nutrient-limited conditions (43). Thus, under limiting conditions, the growth of *P. aeruginosa* and the subsequent production of pyocyanin may alter the microbial community structure by inhibiting the growth of microorganisms sensitive to pyocyanin.

Little is known about how microbial interspecies interactions influence the degradation of crude oil. In this study, an antimicrobial compound, pyocyanin, was isolated and identified from a crude oil-degrading culture containing two strains of *P. aeruginosa*. To test its effect on a microbial community, pyocyanin was added to an oil-degrading microbial community not containing *P. aeruginosa* and the functional diversity of the culture was examined over a 50-day period. Addition of pyocyanin to crude oil-degrading cultures in the laboratory decreased the diversity of the microbial community and resulted in reduced oil degradation.

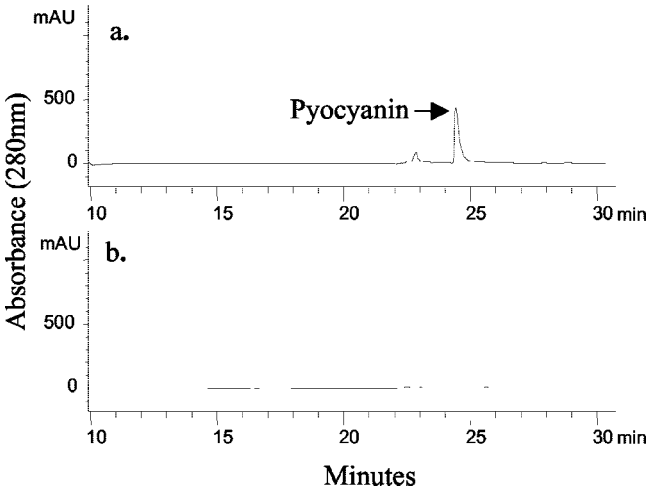

FIG. 1. HPLC analysis of LC and DLC enrichment culture supernatants. LC (a) and DLC (b) oil enrichment cultures were grown for 30 days on either BLC or DBLC oil, respectively. Supernatant was removed and analyzed by preparative reverse-phase HPLC.

MATERIALS AND METHODS

Oil composition. To generate the oil used in this study, *n*-alkane-containing oil (Bonny Light Crude [BLC]; 56% saturates, 31% aromatics, 11% polars, 2% asphaltenes, 35.3° API gravity) was predegraded for 30 days with a coculture of *P. aeruginosa* (20, 31). The remaining oil was extracted three times with dichloromethane, dried over sodium sulfate, and analyzed by gas chromatography (GC) with flame ionization detection (FID). The resulting degraded BLC (DBLC) oil is similar to the parent oil, with the exception that it lacks most of the *n*-alkanes (see Fig. 5a).

Enrichment cultures. The original enrichment cultures (light crude [LC] and degraded LC [DLC]) used in this study were initiated with soil (1 g) from a hydrocarbon-contaminated site in Fairhope, Ala., and basal medium amended with trace metals (BMTM; 25 ml) (20, 21, 23). These cultures were maintained in 125-ml Erlenmeyer flasks with Teflon-lined screw caps for 30 days at 200 rpm and 30°C and have been transferred monthly (4% inoculum) for approximately 80 months (20, 21). The LC enrichment culture was developed on BLC oil, while the DLC enrichment culture was developed on DBLC oil (20). The LC culture is characterized by reduced microbial diversity, the presence of two distinct strains of *P. aeruginosa* (31), and degradation of *n*-alkanes and more complex saturates [i.e., C_{30} 17α(*H*),21β(*H*)-hopane] found in crude oil but reduced aromatic and heterocyclic degradation (9, 20, 21). The DLC culture was observed to have a more diverse microbial community, lacked *P. aeruginosa*, and has been shown to degrade *n*-alkanes, hopanes, aromatics, and heterocyclics. Spectrophotometric analysis (15) of LC and DLC culture supernatants after 30 days of growth on BLC or DBLC oil revealed an average of 9.5 μM pyocyanin in triplicate LC but not DLC culture supernatants. The concentration of pyocyanin observed in our cultures is similar to that observed by others and was the foundation of the studies described below.

Experimental design. To examine the effects of pyocyanin on a crude oil-degrading microbial community, 1% transfers of the original DLC enrichment culture described above were grown in 10 ml of BMTM (23) supplemented with DBLC at 2 mg·ml^{-1} with and without 9.5 μM pyocyanin (Color Your Enzyme, Bath, Ontario, Canada). Analytical controls included tubes containing uninoculated BMTM supplemented with DBLC oil at 2 mg·ml^{-1}. Tubes were shaken in the dark at 200 rpm at 30°C for up to 50 days. At each time point (0, 3, 14, and 50 days), one set of triplicate enrichment cultures was analyzed for microbial community structure and pyocyanin stability while a second set was analyzed for oil degradation as described below. Also, to isolate individual members of the DLC culture, serial dilutions (10^{-5} to 10^{-7}) of the day 50 DLC culture without pyocyanin were plated onto Luria-Bertani agar plates and individual colonies were isolated, grown overnight on Luria-Bertani broth, and stored at −80°C. This isolation step was intended to provide an overview of the microbial community instead of an in-depth characterization of the community diversity. To test for their ability to utilize certain PAHs as growth substrates, isolates were also grown in 50-ml test tubes on 10 ml of BMTM and 100 ppm dibenzothiophene (DBT) and naphthalene (NPH) for 30 days and growth was monitored by serial dilution and plating.

Characterization of pyocyanin. To determine the concentration of pyocyanin in cultures containing *P. aeruginosa*, pyocyanin was isolated and identified from the supernatant of the LC culture after 30 days of growth on BLC oil. Briefly, the supernatant was extracted three times with chloroform and the chloroform layer was further extracted with acidified water (pH 6.0). The acidified water layer (100 μl) was then analyzed by high-performance liquid chromatography (HPLC)–mass spectrometry with an LCQ Finnigan mass spectrometer (MS) equipped with an Agilent 1100 series HPLC system including a UV detector in line (Thermo Finnigan, San Jose, Calif.) (42). Throughout the time course experiment, the stability of pyocyanin was monitored by HPLC (16). Briefly, at each time point, a 5-ml aliquot of cell-free culture supernatant was acidified to a pH of 4.0 with 50% trifluoroacetic acid and filtered (0.2-μm pore size). Samples (100 μl) were injected into a Hewlett-Packard (Palo Alto, Calif.) series 1100 HPLC and separated on a Zorbax 300SB C_{18} column (4.6 mm by 15 cm) with a previously described solvent scheme (16). Samples were monitored at 280 nm with a diode array detector module.

Microbial community structure. At each time point, 1-ml aliquots were removed from triplicate cultures and total community DNA was obtained by cell lysis, phenol-chloroform extraction, and ethanol precipitation by the CTAB (hexadecyltrimethylammonium bromide protocol) (44). From purified DNA, a 323-bp sequence of the V9 region of the 16S rRNA gene was amplified with primers 5570F and 9206GCR (17). Amplification was done with a Techne GeneMate Thermal Cycler (ISC BioExpress, Kaysville, Utah) (30). Following amplification, PCR products were purified with QIAquick PCR purification columns (QIAGEN Inc., Valencia, Calif.). The purified PCR products were then analyzed by denaturing gradient gel electrophoresis (DGGE) with a Dcode universal mutation detection system (Bio-Rad Laboratories, Richmond, Calif.) (30). Briefly, samples were run on an 8% polyacrylamide gel in 1× TAE containing a 40 to 60% denaturing gradient (7 M urea and 40% formamide). Electrophoresis was carried out for 18 h at 50 V and 60°C. The gels were stained for 1 h with SYBER Green I (Molecular Probes, Eugene, Oreg.) and analyzed with a Bio-Rad model 1000 VersaDoc imaging system (Bio-Rad Laboratories).

Bands observed in the DGGE community profile of the DLC culture were excised with a sterile razor blade, and the DNA was eluted overnight into 50 μl of sterile deionized water. The eluted DNA (1 μl) was reamplified and purified as previously described and analyzed on an 8% polyacrylamide gel in 1× TAE containing a 50 to 60% denaturing gradient as described above. Following three rounds of band excision and DGGE analysis, bands were excised and amplified with the primers described above except that primer 9206R lacks the 50-bp GC clamp. Amplified DNA was purified with QIAquick PCR purification columns and sequenced at the Biotechnology Resource Laboratory (Medical University of South Carolina, Charleston) with an ABI 377 DNA sequencer (21). DNA was also extracted from the four individual isolates by the CTAB protocol and compared to community profiles by coelution of bands on DGGE gels and sequence analysis.

Phylogenetic analyses. Sequences were confirmed and hand aligned with the BioEdit sequence alignment editor (22). Sequences were submitted to the advanced BLAST search program (National Center for Biotechnology Informa-

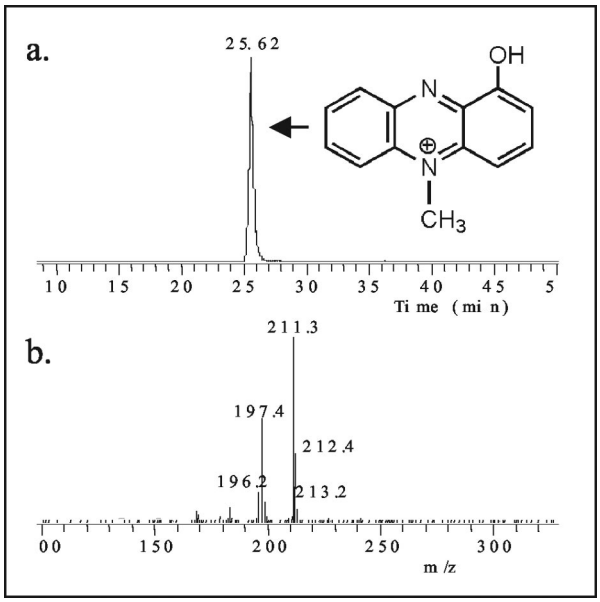

FIG. 2. HPLC (a) and MS (b) analyses of chloroform-acid extracts of LC culture supernatants containing pyocyanin. Liquid chromatography-MS analysis indicates that pyocyanin isolated from the LC culture is identical to the pyocyanin used throughout this study. The inset shows the chemical structure of pyocyanin.

tion) for determination of the most closely related sequences. Multiple-sequence alignment was performed with the ClustalX program (38). The phylogenetic tree was constructed by the neighbor-joining method with PAUP* 4.0b10, and confidence estimates were determined by bootstrap analysis with 1,000 resamplings.

Oil extraction and analysis. At each time point, triplicate cultures were extracted three times with 10 ml of dichloromethane and dried with anhydrous sodium sulfate (12 to 60 mesh; J. T. Baker Chemical Co., Phillipsburg, N.J.). Extracts were evaporated to dryness at 45°C with a rotovap (Buchi R114) under vacuum, and oil residues were shaken with 5 ml of *n*-hexane to precipitate the asphaltene fraction. To monitor *n*-alkane and branched alkane degradation, samples were then analyzed on a Hewlett-Packard series 5890 II Plus gas chromatograph-FID and a 25-m type HP-5 column (0.32-mm diameter, 0.52-μm phase thickness). To monitor PAHs, C_{29} and C_{30} hopanes, and 18α oleanane, the hexane-soluble fraction was also analyzed with a Hewlett-Packard 5890 II gas chromatograph coupled to a 5972A MS (Hewlett-Packard). To better compare the total PAH degradation, a ratio was established between the concentration of PAHs relative to that of 18α-oleanane at each time point. PAHs were normalized to oleanane due to the lack of degradation of oleanane in our crude oil-degrading enrichment cultures (21). Therefore, a reduction in the PAH/oleanane ratio corresponds to a reduction in the total PAH concentration.

Catalase and SOD activities. To analyze catalase and SOD activities, three bacterial strains isolated from the DLC culture (DLC21, -22, and -23) were grown for 24 h as two sets of triplicates on tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.). Growth was monitored spectrophotometrically (optical density at 600 nm), and at the onset of log phase (5 h), 9.5 μM pyocyanin was added to one set of triplicate cultures. At the late-log (13 h) and stationary (21 h) phases, samples (1 ml) were removed from pyocyanin-amended and nonamended cultures and cells were harvested by centrifugation. Cell pellets were resuspended in 500 μl of ice-cold potassium phosphate buffer (50 mM, pH 7.8) and sonicated four times on ice (45). Following centrifugation, sample lysate was removed and stored at −80°C. The total protein concentration in the cell lysates was determined by the Bradford assay (11). Catalase and SOD activities were measured spectrophotometrically at 240 and 550 nm, respectively (26). One unit of catalase degraded 1 μM H_2O_2 per mg of protein per min at 25°C. One unit of SOD inhibited the reduction of cytochrome *c* by 50%.

Nucleotide sequence accession numbers. The partial rRNA gene sequences determined in this study were deposited in the GenBank database under accession numbers AY379761 to AY379766.

RESULTS

Pyocyanin identification. To determine if pyocyanin was present in crude oil-degrading cultures, chloroform-acid extracts of supernatant from the LC and DLC enrichment cultures were analyzed by HPLC fractionation and mass spectroscopy. Analysis of culture extracts after 30 days of growth on oil by reverse-phase HPLC yielded a single UV-absorbing species in the LC but not in the DLC culture extracts (Fig. 1). Further analysis of this peak by mass spectroscopy demonstrated a protonated molecular ion cluster at *m/z* 211/212, thus identifying the peak as pyocyanin (Fig. 2), similar to previous research (42). Pyocyanin isolated from the LC culture demonstrated a mass spectrum identical to that of the pyocyanin standard (data not shown). In addition, throughout the time course experiment, HPLC analysis of the pyocyanin-amended DLC cultures demonstrated that pyocyanin remained stable at an average of 9.5 μM in triplicate cultures over the 50-day incubation period.

Microbial community structure. DGGE profiles were used to examine the overall effect of pyocyanin on microbial community structure. At day 3, the DGGE profile of the DLC enrichment culture without pyocyanin showed six observable bands (B1 to B6; Fig. 3a). The profile of the DLC culture

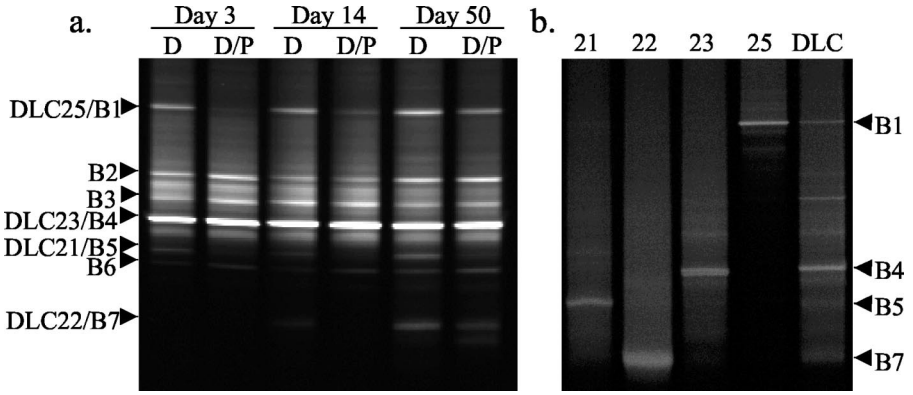

FIG. 3. (a) DGGE analysis of the DLC enrichment cultures without (D) and with (D/P) pyocyanin amendment at 3, 14, and 50 days of growth. B1 to B7 point to bands that were further excised and sequenced. (b) Individual isolates (DLC21, -22, -23, and -25) were compared to the DLC community profile and aligned with its corresponding DGGE band. All lanes are representative of triplicate samples.

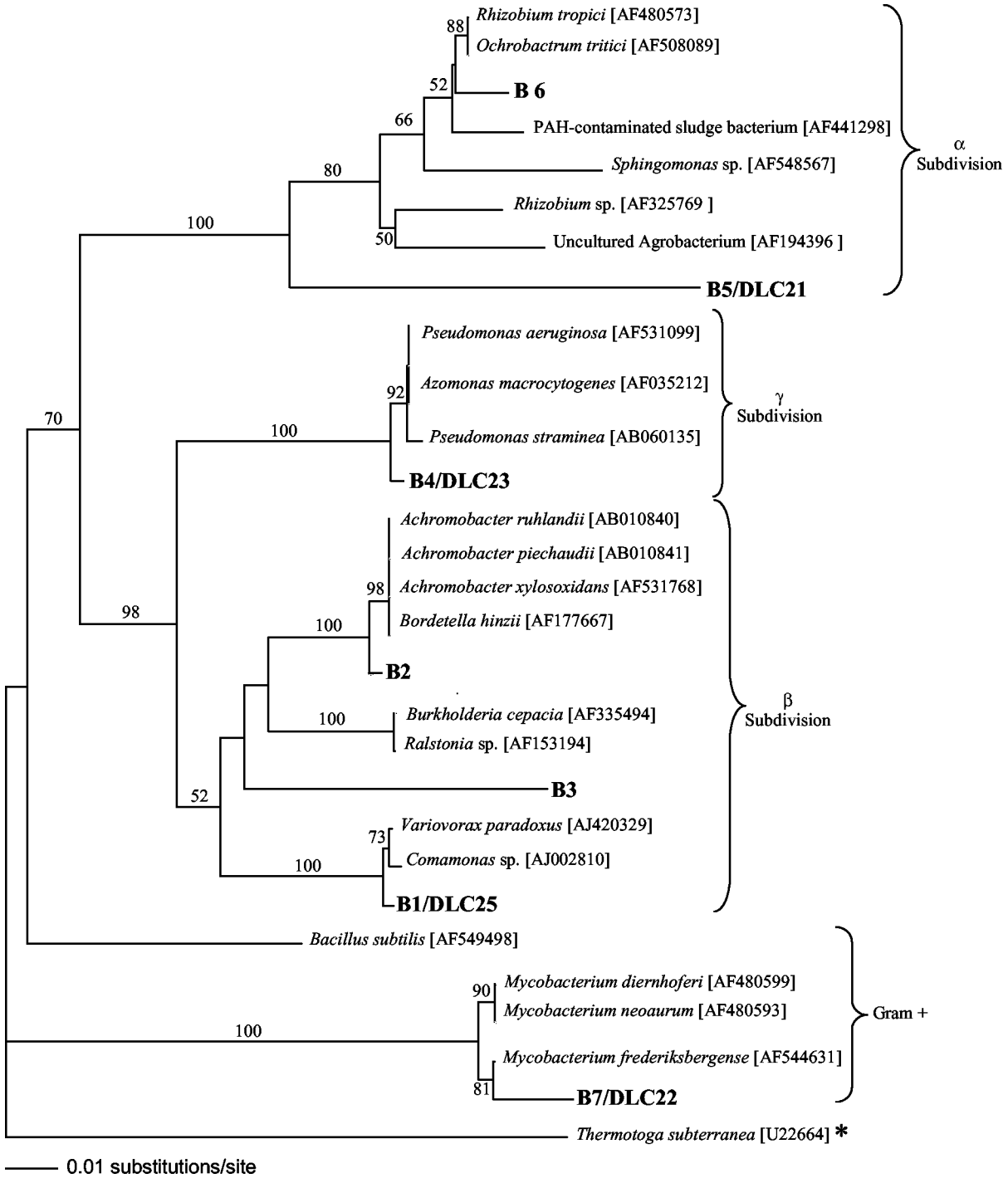

FIG. 4. Phylogenetic tree, constructed by the neighbor-joining method, showing the relationship of bands excised from DGGE gels to 16S rRNA gene sequences of previously described bacteria. Numbers represent confidence estimates determined by bootstrap analysis with 1,000 replicates. *Thermotoga subterranea* was used as the outgroup.

amended with pyocyanin showed four bands that are similar to those of the unamended culture (B2, B3, B4, and B6), while two bands (B1 and B5) were not resolved. The band between B2 and B3 is a result of nonspecific amplification of B4 and is observed in both pyocyanin-amended and unamended cultures. After 14 days, the banding profile of the unamended culture remained similar to that seen on day 3, with the exception of the appearance of a new band (B7), which was not apparent in the pyocyanin-amended culture. Also, at day 14, the faint appearance of B1 was observed in the pyocyanin-amended cultures. At 50 days, the unamended DLC culture remained stable, demonstrating seven distinct bands (B1 to B7). After 50 days, all bands, with the exception of B5, were observed in the pyocyanin-amended cultures. Serial dilution

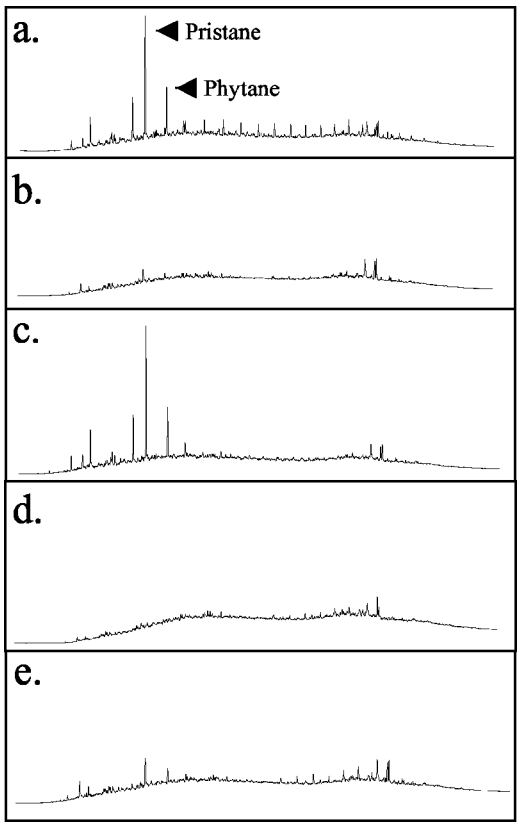

FIG. 5. GC-FID traces of DBLC oil extracted after 14 and 50 days of incubation with the DLC enrichment culture with and without pyocyanin amendment. (a) Uninoculated DBLC control at 14 days. (b) DBLC remaining after 14 days of incubation with the DLC culture without pyocyanin. (c) DBLC remaining after 14 days of incubation with the DLC culture amended with pyocyanin. (d) DBLC remaining after 50 days of incubation with the DLC culture without pyocyanin. (e) DBLC remaining after 50 days of incubation with the DLC culture amended with pyocyanin. All traces are representative of triplicate cultures. *x* axes represent time (0 to 25 min); *y* axes represent detector response (1,000 to 8,000).

and plating of the DLC cultures with and without pyocyanin resulted in the isolation of four morphologically distinct members of the microbial community. DNAs extracted from these isolates (DLC21, -22, -23, and -25) were compared to community profiles and aligned with corresponding DGGE bands (Fig. 3b).

Phylogenetic analysis of the partial 16S rRNA gene sequence of DGGE bands and isolates showed that the crude oil-degrading microbial community members are distributed among a wide range of taxonomic groups (Fig. 4). Microorganisms represented by bands B1 to B6 are distributed among the alpha, beta, and gamma subclasses of the class *Proteobacteria*, while the microorganism represented by band B7 was found to be a member of the high-G+C subgroup of the gram-positive genera. Furthermore, sequence analysis demonstrated the similarity of isolates to their corresponding DGGE bands.

Crude oil degradation. To examine if pyocyanin affects the DLC culture's ability to degrade DBLC, oil extracted at different time points was analyzed by GC-FID (Fig. 5). Compared to the uninoculated DBLC control (Fig. 5a), GC-FID traces demonstrated that after 14 days, the DLC culture without pyocyanin was capable of degrading many of the saturates in DBLC, including the branched alkanes, pristane, and phytane (Fig. 5b). However, the DLC culture amended with pyocyanin demonstrated a decreased ability to degrade the oil components, as shown by the remaining pristane and phytane peaks (Fig. 5c). At 50 days, many saturates were still present in the DLC culture containing pyocyanin (Fig. 5e) compared to the DLC culture alone (Fig. 5d).

GC-MS analysis was used to quantify a range of compounds not resolved by GC-FID, including 53 PAHs including heterocycles, 18α-oleanane, and C_{29} and C_{30} hopanes, and the amounts of each compound remaining in the culture after 14 and 50 days were determined. The DLC culture without pyocyanin had significantly ($P < 0.05$, analysis of variance) reduced PAH/18α-oleanane ratios at 14 and 50 days (7.5 and 6.3, respectively) compared to those of the pyocyanin-amended culture (9.7 and 8.6, respectively) (Table 1). This suggests that the presence of pyocyanin in the DLC culture reduces PAH degradation. While no difference was observed in the degradation of most of the crude oil compounds analyzed by GC-MS, the degradation of three compounds, DBTs, NPHs, and hopanes, was decreased in the presence of pyocyanin (Table 1). After 14 days, the DLC culture had, on average, a 16% reduction in DBT compounds compared to the control, while the DLC culture amended with pyocyanin remained similar to

TABLE 1. Ratios of PAHs to oleanane and concentrations of DBTs, NPHs, and hopanes remaining after 14 and 50 days of incubation with pyocyanin-amended or unamended DLC enrichment cultures

Incubation time and culture	PAH/ oleanane ratio[a]	Concn (ng ml of oil^{-1}) ± SEM[b]									
		DBT	C_1 DBT	C_2 DBT	C_3 DBT	C_1 NPH	C_2 NPH	C_3 NPH	C_4 NPH	C_{29} hopane	C_{30} hopane
14 days											
DBLC[c]	9.5 ± 0.7	41 ± 1	196 ± 5	233 ± 15	179 ± 6	28 ± 14	527 ± 118	1,495 ± 145	1,315 ± 5	849 ± 63	1,245 ± 105
D[d]	7.5 ± 0.9	36 ± 8	182 ± 5	167 ± 34	157 ± 15	1 ± 1	51 ± 13	533 ± 66	742 ± 51	939 ± 42	1,380 ± 40
D/P[e]	9.7 ± 0.7	55 ± 0	226 ± 15	229 ± 5	174 ± 5	13 ± 6	380 ± 84	1,225 ± 105	1,225 ± 5	773 ± 63	1,165 ± 85
50 days											
DBLC	8.1 ± 0.8	47 ± 4	222 ± 2	264 ± 2	173 ± 8	34 ± 30	538 ± 289	1,314 ± 346	1,198 ± 203	863 ± 8	1,390 ± 10
D	6.3 ± 1.1	27 ± 1	168 ± 6	181 ± 6	178 ± 12	1 ± 1	108 ± 54	816 ± 178	1,041 ± 160	389 ± 14	730 ± 3
D/P	8.6 ± 0.6	32 ± 5	157 ± 5	164 ± 14	148 ± 8	20 ± 17	434 ± 206	1,395 ± 365	1,445 ± 245	938 ± 93	1,500 ± 100

[a] Concentrations of PAHs were normalized to oleanane as a measure of PAH degradation.
[b] The data are for triplicate samples. C_1 to C_4 represent carbon numbers of alkyl groups in alkylated homologues.
[c] Uninoculated control.
[d] Unamended DLC cultures.
[e] Pyocyanin-amended DLC cultures.

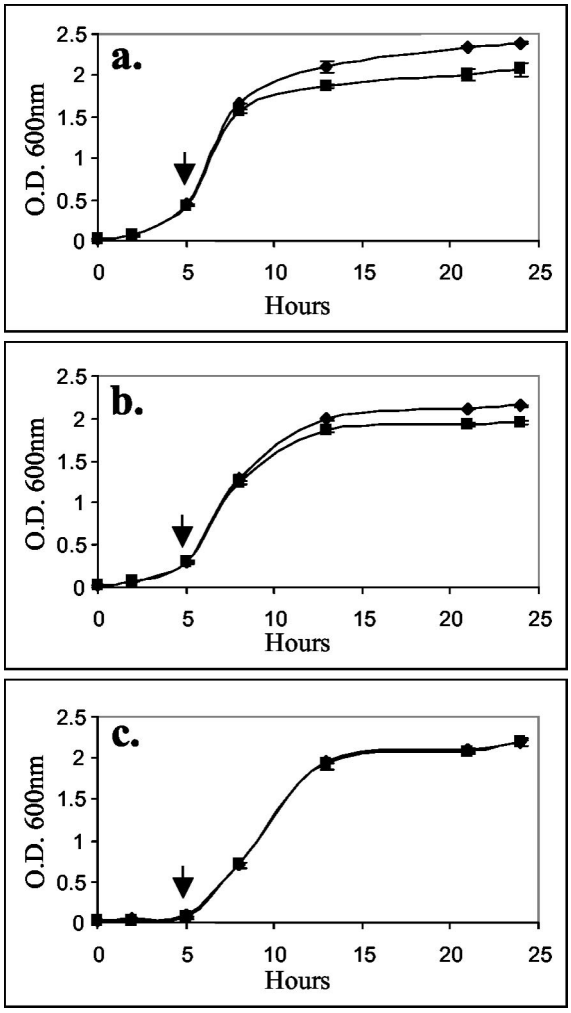

FIG. 6. Growth of DLC isolates on TSB with (◆) and without (■) pyocyanin amendment. Panels: a, DLC21; b, DLC22; c, DLC23. DLC25 demonstrated no growth on TSB and was not examined. The arrow at 5 h indicates the time of pyocyanin amendment of one set of cultures. All points represent the average and standard error of a triplicate series. O.D., optical density.

the control. However, after 50 days, both the DLC cultures with and without pyocyanin had decreased concentrations of DBTs compared to the control (29 and 22% reductions, respectively). The second class of compounds affected by the presence of pyocyanin was the NPHs. At days 14 and 50, GC-MS analysis demonstrated decreased concentrations of NPHs remaining in the DLC cultures without pyocyanin (61 and 36%, respectively) while the concentrations in the pyocyanin-amended cultures remained similar to those in the controls. After 14 days, no substantial degradation of either C_{29} or C_{30} hopane was demonstrated by the DLC cultures with and without pyocyanin compared to that of the control. However, after 50 days, the DLC culture without pyocyanin contained decreased concentrations of both C_{29} and C_{30} hopanes (55 and 48%, respectively) while the DLC culture amended with pyocyanin remained similar to the control. The individual isolates from the DLC culture (DLC21, -22, -23, and -25) demonstrated no growth on either DBT or NPH.

Catalase and SOD activities. When they were grown on TSB and amended with 9.5 μM pyocyanin at the onset of log phase, the growth of isolates DLC21, -22, and -23 was affected to various degrees compared to that of unamended cultures. DLC25 was unable to grow on TSB, so it was not tested further. The final cell densities of DLC21 and DLC22 were significantly ($P < 0.01$, analysis of variance) reduced, by 15 and 10%, respectively, when they were amended with pyocyanin, while the cell density of DLC23 was not affected (Fig. 6). Furthermore, protein extracts from the three isolates demonstrated different catalase and SOD activity levels (Table 2). Isolate DLC21 demonstrated the lowest activity levels for both catalase and SOD, while isolate DLC23 demonstrated the highest levels of activity in both assays. Compared to DLC21 and -23, DLC22 demonstrated intermediate catalase and SOD enzyme activity levels. In this study, individual isolates demonstrated no growth on crude oil, thus requiring pyocyanin inhibition to be measured under nutrient-rich conditions. While isolates DLC21 and -22 demonstrated reduced growth in the presence of pyocyanin under these conditions, it is possible that pyocyanin would have an increased effect on these members of the microbial community grown on crude oil.

DISCUSSION

Biodegradation of crude oil is a complex process requiring a metabolically diverse microbial community. Throughout this process, the microbial community profile often shifts corresponding to the degradation of each oil fraction (2). For example, during the degradation of *n*-alkanes, which are among the first compounds attacked, bacteria within the oil-degrading community possessing group 1 alkane hydroxylase genes are first selected, providing for the degradation of short-chain alkanes (34). This selection is often followed by the growth of bacteria possessing group 2 and 3 alkane hydroxylase genes and capable of degrading long-chain alkanes. This suggests that upon initial exposure to crude oil, the microbial community profile would shift toward microorganisms, such as *P. aeruginosa*, capable of degrading the *n*-alkane component of crude oil. This population shift may establish either beneficial or antagonistic interspecies interactions that ultimately result in altered overall community degradation potential. For example, we have previously demonstrated that the presence of *P. aeruginosa* in crude oil-degrading enrichment cultures decreases microbial diversity and aromatic degradation (20).

In this study, the antibacterial compound pyocyanin was identified by liquid chromatography-MS from the supernatant of a crude oil-degrading enrichment culture containing two

TABLE 2. Catalase and SOD activities in extracts of DLC isolates

Isolate	Mean enzyme activity (U/mg of protein) ± SEM[a]	
	Catalase	SOD
DLC21	39 ± 1	29 ± 2
DLC22	285 ± 8	77 ± 10
DLC23	343 ± 8	129 ± 12

[a] Data are for triplicate samples. At the onset of log phase (5 h), cultures were supplemented with 9.5 μM pyocyanin.

distinct strains of *P. aeruginosa* (31), and it was further demonstrated that pyocyanin reached a concentration of 9.5 μM in the culture supernatants. While the direct mechanism of pyocyanin-induced bactericidal activity remains unclear, previous research has demonstrated bacterial toxicity to micromolar concentrations of pyocyanin (5, 24). For instance, when the medium was amended at mid-exponential phase with pyocyanin at the same concentration used in our studies (9.5 μM), the growth and viability of the gram-positive bacterium *Micrococcus luteus* were significantly reduced (5). Also, Hassan and Fridovich demonstrated that the generation time of *Escherichia coli* cells growing on glucose-minimal medium increased from 43 to 145 min when they were supplemented with 10 μM pyocyanin (24). Therefore, the concentration of pyocyanin found in our crude oil-degrading enrichment culture could influence pyocyanin-sensitive members of the microbial community.

Analysis of the DLC enrichment culture with and without pyocyanin amendment over a 50-day incubation period demonstrated that the presence of pyocyanin altered the composition of the community. DGGE analysis and single-isolate studies revealed that individual members of the community exhibited various degrees of sensitivity to pyocyanin. For example, the growth of isolates DLC21, -22, and -25 was suppressed by the presence of pyocyanin while the growth of DLC23 was not affected. Various levels of susceptibility to pyocyanin have been reported for individual microorganisms, and susceptibility to pyocyanin is thought to depend on the rate of pyocyanin uptake and the level of antioxidant enzyme (SOD and catalase) activity (5, 24, 25, 27, 33, 40). For example, pyocyanin toxicity has been shown to decrease when *E. coli* cells are grown on nutrient-rich medium that supports higher catalase and SOD activity levels. Also, *P. aeruginosa* isolates are known to increase antioxidant activity when grown under conditions stimulating pyocyanin production (low nutrient), resulting in cellular protection (25). Similarly, in this study, DLC23 contained the highest catalase and SOD activity levels of the three bacteria isolated from our crude oil-degrading cultures and was not sensitive to pyocyanin. While it is currently assumed that differential expression of catalase and SOD activities is the principal means of pyocyanin resistance, it is also possible that other general antibiotic resistance mechanisms play a role in pyocyanin resistance (5).

The presence of pyocyanin not only affected the composition of the community but also altered the degradation capability of the culture. In pyocyanin-amended cultures, the degradation of DBTs was decreased at day 14 but not at day 50 compared to that in unamended cultures. While it is unable to grow on DBT alone, it is interesting that the growth of DLC22 was suppressed early during the time course and corresponded to reduced degradation of DBTs. However, after 50 days, DLC22 was observed in the pyocyanin-amended cultures, which may correspond to the increased degradation of DBTs. The ability of DLC22 to overcome complete inhibition by pyocyanin could be related to an increase in the catalase and SOD activity levels. Furthermore, DLC21 contains the lowest catalase and SOD activities of the isolates tested and is not observed in DGGE profiles of pyocyanin-amended cultures throughout the time course. Also, while catalase and SOD activities were not available for DLC25, DGGE analysis indicates that this isolate appears to be moderately sensitive to pyocyanin, with growth suppressed only early in the time course. It is possible that these pyocyanin-sensitive bacteria are responsible for the degradation or partial transformation of the aromatic and complex saturates examined in this study.

The microbial interactions occurring during crude oil degradation are not well understood and most likely include numerous positive and negative interactions. For example, toxicity of PAHs, such as NPH, to certain bacteria has been observed (10). Furthermore, degradation of PAHs by individual isolates often does not mirror PAH degradation in complex mixtures such as crude oil (12, 35). In these complex contaminant mixtures, partial transformation of PAHs, often occurring through what has been described as a cometabolic process (7, 36), is more likely to occur (13). Thus, while the degradation of DBTs, NPHs, and hopanes has not yet been linked to the isolates examined in this study, the decreased degradation of these compounds in the presence of 9.5 μM pyocyanin suggests that pyocyanin-sensitive bacteria may be involved in their partial or complete degradation.

While much is known about the effects of *P. aeruginosa*-produced factors in clinical settings, much less is known about how they influence microbial community interactions within environmental settings. In this study, pyocyanin was demonstrated to influence the functional diversity of a crude oil-degrading enrichment culture. While pyocyanin is one of many factors that could influence community diversity and degradation potential, the presence of this factor in our cultures resulted in reduced microbial diversity, as well as reduced overall community degradation capability. This represents one of the first studies to examine how pyocyanin production by an individual member of a crude oil-degrading microbial community can influence the overall composition and function of that community. Lastly, the data suggest the importance of understanding how environmental contaminants impact interspecies interactions within a microbial community during a bioremediation process.

ACKNOWLEDGMENTS

This research was supported in part by a grant from the South Carolina Sea Grant Consortium. Funding for T.J.M. was provided through the Superfund Basic Research Program (grant P42 ES04917).

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